

## Environmental detection of mouse allergen by means of immunoassay for recombinant Mus m 1

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**Background:** Mouse urinary allergens are an important cause of occupational asthma in animal facilities. Domestic exposure to mouse allergens is a risk factor for asthma among inner-city residents.

**Objective:** We sought to develop a sensitive and specific assay for assessing environmental mouse allergen exposure.

**Methods:** An ELISA for recombinant (r)Mus m 1 was developed by using rabbit polyclonal antibodies to rMus m 1 that were affinity purified against the natural allergen. Assay specificity was established by means of immunoblotting and ELISA. Mus m 1 levels in mouse, other mammalian allergenic products, and house dust samples from inner-city homes were compared.

**Results:** Polyclonal antibodies to Mus m 1 showed a single 20-kd band on immunoblots against rMus m 1 and male mouse urine. Parallel dose-response curves were obtained by using mouse urine extract and natural Mus m 1 or rMus m 1. Mus m 1 was detected in mouse allergenic products (0.10-10.0 µg/mL) and in gerbil allergenic products (0.1 µg/mL) but was less than the limit of detection in epithelial extracts from 10 other animal species. Environmental measurements showed an excellent correlation between Mus m 1 levels in house dust extracts from inner-city asthma studies by using 2 different Mus m 1 standards ( $n = 22$ ;  $r = 0.99$ ;  $P < .001$ ).

**Conclusions:** A highly sensitive ELISA has been developed with rMus m 1. This assay is suitable for monitoring domestic and environmental exposure to mouse urinary allergens. (*J Allergy Clin Immunol* 2004;114:341-6.)

**Key words:** Mouse allergen, occupational asthma, in vitro diagnostics, allergen standardization, immunoassay, recombinant allergen, inner-city asthma

Personal exposure to mouse urinary proteins (MUPs) is a well-established cause of occupational allergic disease among laboratory animal technicians and animal handlers in industry and in academic research centers. Inhalation of mouse proteins results in IgE-mediated sensitization and symptoms of rhinitis, asthma, and, in some cases, dermatitis. Workers most at risk for development of occupational allergic diseases are those who receive the highest levels of exposure from handling animals, bedding, cages, or cage-washing systems.<sup>1-6</sup> Recently, sensitization to mouse allergens has also been identified as a risk factor for the development of asthma among inner-city children who were exposed to high levels of allergen in their homes.<sup>7,8</sup>

The major mouse allergens are secreted in large amounts with the urine of male mice (1-5 mg/mL) and are a group of proteins known as MUPs. The MUPs are lipocalins that are excreted under hormonal control by male mice.<sup>9-11</sup> Deposition of these pheromone-binding proteins allows dominant male mice to recognize other individuals and to mark their territories.<sup>12</sup> The MUPs occur as multiple isoforms, and the 3-dimensional structure of one of these isoforms has been determined by means of x-ray crystallography and nuclear magnetic resonance spectroscopy.<sup>11,13-15</sup> In the allergen nomenclature the MUP complex is described as Mus m 1 to reflect that MUP is a major mouse allergen.<sup>3,16</sup>

Previous studies have used a variety of assays for measuring environmental exposure to rodent urine allergens, including RAST inhibition, and sandwich or inhibition ELISA for urinary proteins.<sup>17-20</sup> These assays typically used polyclonal antibodies and showed varying degrees of sensitivity and cross-reactivity between mouse and rat urinary proteins. A multicenter study carried out in The Netherlands, the United Kingdom, and Sweden showed dramatic differences between the results of RAST inhibition and ELISA (>1000-fold) and smaller but nonetheless significant differences between ELISA for urine allergens. This study highlighted the need for improved standardization of assay protocols, including dust and air sampling procedures, use of antibodies of defined specificity, and development of allergen standards

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Supported in part by a cofinancing research grant to AS from the Ministero Industria Università e Ricerca, Italy (COFIN MURST).

Disclosure of potential conflict of interest: E. Ferrari—none disclosed. A. Tsay is employed by INDOOR Biotechnologies, Inc. P. A. Eggleston—none disclosed. A. Spisni—none disclosed. M. D. Chapman is president of INDOOR Biotechnologies, Inc.

Received for publication March 8, 2004; revised April 13, 2004; accepted for publication April 19, 2004.

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doi:10.1016/j.jaci.2004.04.028

**Abbreviations used**

MUP: Mouse urinary protein  
PBS-T: PBS, pH 7.4 containing 0.05% Tween 20

of known absolute concentration.<sup>21,22</sup> In the United States an enzyme immunoassay for Mus m 1 developed by Ohman et al<sup>3</sup> was used to assess occupational allergen exposure in mouse breeding facilities. This assay was also used in the inner-city asthma studies, which measured allergen levels in homes in 7 US cities.<sup>7,8</sup> However, the antibodies used in those studies are not available, and the assay is no longer in general use.

We have developed a specific assay for Mus m 1 using polyclonal antibodies raised against recombinant (r)Mus m 1, which had been produced in *Pichia pastoris*, a yeast expression vector that expresses rMus m 1 at high levels into culture medium.<sup>23</sup> This assay shows high sensitivity, and the results were validated by comparison with previous exposure assessments of Mus m 1. The results suggest that the Mus m 1 ELISA will enable exposure to mouse allergens to be accurately monitored in both occupational and domestic environments. This will allow risk levels associated with sensitization and allergic disease to be established and the effects of environmental control procedures to be objectively evaluated.

**METHODS****Purified Mus m 1**

A recombinant isoform of the Mus m 1 complex (entry name at EMBL Nucleotide Database MMU 309921; EMBL Sequence Accession no. AJ 309921) was expressed in *P pastoris* and purified by means of HPLC, as described previously.<sup>23</sup> Briefly, the culture supernatant was applied to an ion-exchange column (SOURCE 15Q 4.6/100, Amersham Biosciences) equilibrated with 10 mmol/L Tris-HCl, pH 7.2, and eluted with a 100 to 600 mmol/L NaCl gradient. The rMus m 1 peak was further purified by means of size exclusion (Sephacryl S200, HiPrep 16/60 column), dialyzed, and stored at 4°C. Purified rMus m 1 (lot 2621) produced a single band on SDS-PAGE (>90% purity). The molecular mass of rMus m 1 was determined by means of electrospray mass spectrometry with an Applied Biosystems-SCIEX instrument, model API 365. For this measurement, we used 100  $\mu$ L of rMus m 1 solution (44  $\mu$ mol/L in 10 mmol/L Tris-HCl, pH 7.2) diluted with an equal volume of 50% acetonitrile and with the addition of 2  $\mu$ L of acetic acid.

A natural (n)Mus m 1 standard (MUP E428) that had been used in previous studies was used as an allergen reference for ELISA.<sup>7,8</sup> This standard had a concentration of 5.65 mg/mL nMus m 1. A second preparation of nMus m 1 was purified from 10-fold-concentrated mouse urine by means of gel filtration HPLC with a Sephacryl S200 column equilibrated in 0.05 mol/L sodium phosphate and 0.5 mol/L NaCl, pH 7.4. Eluted fractions were analyzed by means of ELISA, and the nMus m 1 peak was pooled and concentrated. The purified nMus m 1 (lot 2630) showed 2 isoforms at 18 and 20 kd and was 95% pure as judged by means of silver-stained SDS-PAGE.

**Polyclonal rabbit antibodies to Mus m 1**

New Zealand rabbits were immunized with 1 mg of rMus m 1 in complete (first injection) or incomplete Freund adjuvant (4 subcutaneous injections over a 9-week period). The polyclonal rabbit

antisera had a titer of greater than 1:100,000 in ELISA by using microtiter plates coated with mouse urine. Antisera were tested for binding to rMus m 1 and to mouse urine by means of immunoblotting. Samples of rMus m 1 or urine in 12 mmol/L Tris-HCl, pH 6.8, containing 0.4% SDS and 2-mercaptoethanol, were heat denatured and separated by means of electrophoresis on 12% SDS-polyacrylamide slab gels. The proteins were transferred to a nitrocellulose membrane (Hybond, Amersham Biosciences) by means of electroblotting at 100 mV for 2 hours. The membranes were blocked and incubated with polyclonal anti-Mus m 1 diluted 1:1000. Bound antibodies were detected with peroxidase-conjugated anti-rabbit IgG diluted 1:2000, and reactivity was developed by means of chemiluminescence with an ECL kit (Amersham Biosciences).

**ELISA for quantification of Mus m 1**

The IgG fraction of the anti-Mus m 1 antibody was purified by means of affinity chromatography with recombinant protein G and used for allergen capture in ELISA. Polyclonal anti-Mus m 1 antibodies were also affinity purified over an immunosorbent column prepared by coupling concentrated mouse urine to cyanogen bromide-activated sepharose. Antibodies were eluted with 0.1 mol/L glycine (pH 3.0), dialyzed against PBS, and concentrated. The affinity-purified antibodies were biotinylated with EZ-Link Sulfo-NHS-LC (Pierce) and used for allergen detection. The Mus m 1 assay was modified from previously published ELISA techniques.<sup>24</sup> Microtiter plates (NUNC Maxisorp) were coated with 100 ng/well IgG anti-Mus m 1 in 50 mmol/L carbonate-bicarbonate buffer, pH 9.6, and incubated overnight at 4°C. After washing with PBS/0.05% Tween 20 (PBS-T), the plates were incubated for 30 minutes with 1% BSA-PBS-T followed by diluted allergen extracts or house dust extracts (1 hour). Bound Mus m 1 was detected by adding 0.1 mL of a 1:1000 diluted biotinylated anti-Mus m 1 (1 hour), and the assay was developed by adding 2,2'-azino-di-(3 ethylbenzthiazoline sulfonic acid) substrate solution.

The assay was quantified by using a control curve of doubling dilutions of a mouse urine sample that contained from 0.05 to 25 ng/mL Mus m 1 (IBI 2508). Results for allergen extracts or house dust extracts were interpolated from the linear part of the curve. The Mus m 1 standard was substandardized by means of ELISA against the nMus m 1 allergen standard (MUP E428) that had been used previously.<sup>7,8</sup> The Mus m 1 concentration determined by means of ELISA was further verified by comparison with the protein content of purified rMus m 1, the concentration of which had been calculated from absorption measurements by using the extinction coefficient at 276 nm ( $\epsilon = 11,345 \text{ M}^{-1} \text{ cm}^{-1}$ ) calculated on the basis of rMUP sequence. The concentrations of serial dilutions of rMus m 1 (from 0.049-3.125 ng/mL) were determined by means of ELISA and OD<sub>276</sub>.

The specificity of the Mus m 1 assay was evaluated by comparing allergen levels in 24 epithelia and hair extracts from 12 mammalian species: mouse, rat, gerbil, cat, dog, cow, horse, hog, goat, guinea pig, hamster, and rabbit. These products were obtained from 5 commercial manufacturers: ALK-ABELLO, Roundrock, Tex; Allergy Labs of Oklahoma, Oklahoma City, Okla; Antigen Laboratories, Liberty, Mo; Greer Laboratories, Lenoir, NC; and Nelco Laboratories, Deer Park, NY.

**Environmental allergen measurements**

The ELISA was validated by comparing Mus m 1 levels in house dust samples that had previously been assayed by using the MUP E428 standard.<sup>7,8</sup> The house dust extracts were collected in Baltimore, Md, as part of studies of mouse allergen exposure and inner-city asthma.<sup>7,8</sup> Twenty house dust extracts were assayed at dilutions ranging from 1:10 to 1:80, and the results of the 2 Mus m 1 allergen standards were compared by using linear regression analysis with Microsoft Excel software.

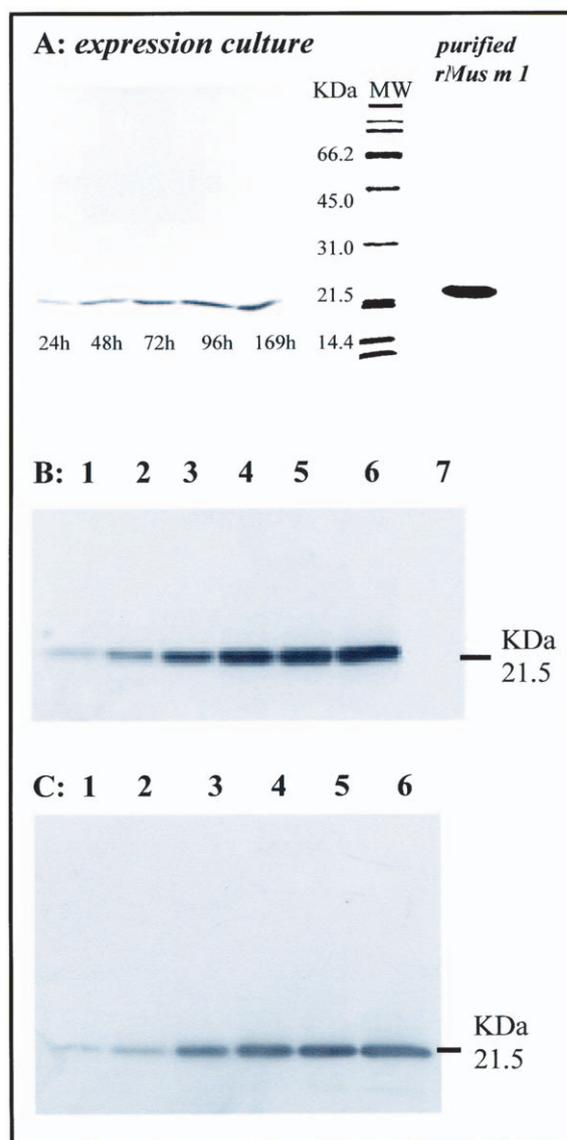
## RESULTS

### Polyclonal antibodies to rMus m 1

Taking advantage of the fact that *P pastoris* secretes very low levels of intrinsic proteins, the production of rMus m 1 in minimal growth medium was an effective strategy to obtain a high yield of pure and homogeneous allergen.<sup>23</sup> The SDS-PAGE analysis of the yeast supernatant showed no bands other than that corresponding to rMus m 1 (Fig 1, A). After purification by means of HPLC, the molecular mass of rMus m 1, determined by using electrospray mass spectroscopy, was 18,713d, which is in agreement with the theoretic value of 18,710d. This protein was used to obtain the polyclonal rabbit anti-Mus m 1 antibodies. The specificity of the antibodies was analyzed by means of immunoblotting with purified Mus m 1 and urine collected from dominant male mice, which are known to excrete up to 5 mg/mL of the Mus m 1 protein complex in urine. The polyclonal rabbit antibody reacted strongly against rMus m 1 on immunoblotting, producing a single band with an apparent molecular weight of approximately 20 kd, and could detect as little as 0.5 ng of recombinant protein (Fig 1, B). Similarly, only one band of that molecular weight was detected in untreated male urine diluted to approximately the same rMus m 1 concentration (Fig 1, C). A recombinant green fluorescent protein used as a negative control showed no reaction with the rabbit anti-Mus m 1 antibody (Fig 1, B, lane 7). These results showed that the polyclonal antibodies reacted with a single protein in male mouse urine (ie, Mus m 1).

### Quantitative analysis of Mus m 1

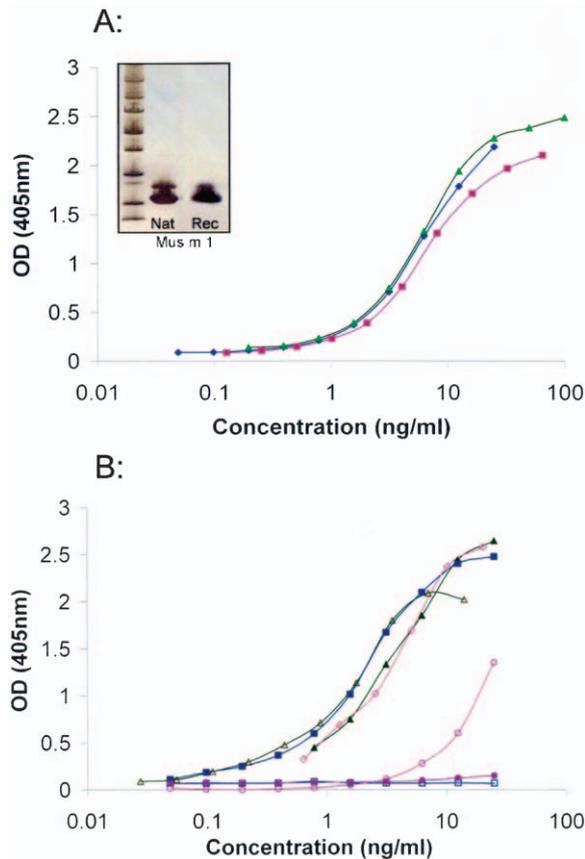
A 2-site ELISA was established by using the IgG fraction of rabbit polyclonal antibodies to Mus m 1 for allergen capture and biotinylated affinity-purified anti-Mus m 1 antibodies for detection. The assay was highly sensitive (limit of detection, 400 pg/mL), and parallel dose-response curves were obtained by using the mouse urine standard (IBI 2508), purified rMus m 1, and nMus m 1 (Fig 2, A). Parallel dose-responses curves were also obtained by using commercial mouse allergen extracts (Fig 2, B). The Mus m 1 assay was calibrated by using a control curve of concentrated mouse urine (IBI 2508) that had been substandardized against a purified preparation of nMus m 1 used in previous studies (MUP E428). The goal of this substandardization was to ensure consistency with previously published mouse allergen exposure data on allergen levels in inner-city homes. Mus m 1 levels in 22 house dust extracts were compared by using the 2 standards to validate the relationship between these standards. Linear regression analysis showed an excellent quantitative correlation between the Mus m 1 levels of the dust extracts by using the MUP E428 and IBI 2508 standards (Fig 3). Intra-assay and interassay variability were assessed by measuring Mus m 1 in 20 house dust extracts. The intra-assay variation, calculated by assaying the 20 samples 3 times in one assay, was 6.1%. The mean interassay coefficient of variation,



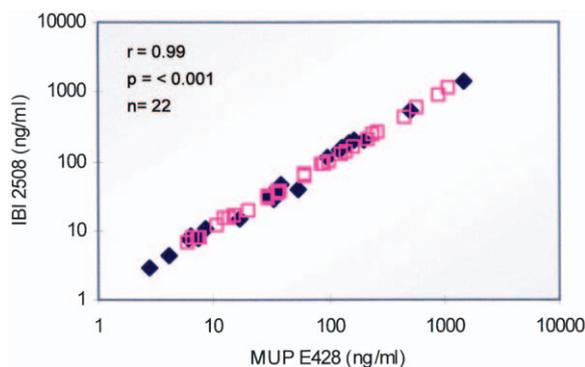
**FIG 1.** Specificity of polyclonal anti-Mus m 1 antibody. **A**, SDS-PAGE of yeast culture supernatant and purified rMus m 1. **B**, Immunoblot showing polyclonal rabbit anti-Mus m1 antibody binding to 0.5, 1, 3, 7, 10, and 15 ng of rMus m 1 (lanes 1-6) and green fluorescent protein (lane 7). **C**, Dominant male mouse urine at similar Mus m 1 concentrations (lanes 1-6).

obtained by assaying the 20 samples on 3 separate days, was 14.4%.

A further validation of the ELISA procedure was obtained from a plot that compared rMus m 1 concentrations extrapolated from a standard curve with the values measured on the basis of absorption measurements at a wavelength of 276 nm (Fig 4). For rMus m 1 concentrations in the range of 0.195 to 3.125 ng/mL measured from its OD at 276 nm, the plot is linear, with a slope close to 0.5 and an  $r^2$  value of 0.99, suggesting that the actual Mus m 1 concentrations in the MUP E428 and IBI 2508 standards are 2-fold lower than the determinations made by using the extinction coefficient.



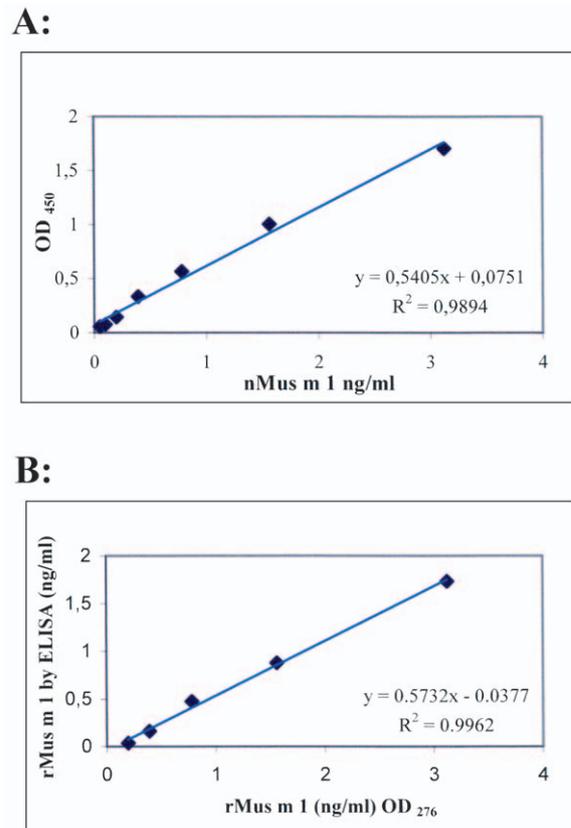
**FIG 2.** Mus m 1 dose-response curves. **A**, Dose-response curves with mouse urine standard (IBI 2508; ♦), rMus m 1 (lot 2621; ▲), and nMus m 1 (lot 2630; ■). *Inset*, SDS-PAGE of nMus m 1 and rMus m 1. **B**, Mus m 1 dose-response curves with commercial allergenic products from mouse (▲ and ◇), rat (□), hamster (●), and gerbil (○) and for IBI 2508 (■) and the nMus m 1 primary reference MUP E424 (Δ).



**FIG 3.** Mus m 1 levels in house dust extracts ( $n = 22$ ) were analyzed by means of ELISA on 2 different days (♦ and □) by using either MUP E428 or IBI 2508 Mus m 1 standard.

### Specificity of Mus m 1 ELISA

The assay showed no cross-reactivity with 10 animal hair or epithelial extracts from rat, cat, dog, guinea pig, hamster, rabbit horse, goat, hog, and cow (Fig 2, B, and Table I). The only observed cross-reactivity was with 2



**FIG 4.** **A**, Mus m 1 ELISA standard curve. **B**, Quantitative recovery of purified rMus m 1: concentrations of rMus m 1 extrapolated from the standard curve (Fig 4, A) and values determined by means of absorption measurements at 276 nm.

gerbil epithelial extracts that showed a consistently low level of reactivity in the assay. Comparison of Mus m 1 levels in diagnostic allergenic products from US allergen manufacturers showed that Mus m 1 levels ranged from 0.17 to 10.02  $\mu\text{g/mL}$  (geometric mean, 0.57  $\mu\text{g/mL}$ ; Table I). By contrast, the Mus m 1 level in a mouse urine sample was 4550  $\mu\text{g/mL}$ , as would be expected on the basis of previous studies.<sup>9-11</sup>

The apparent cross-reactivity with gerbil was investigated by comparing amino acid sequence homology between Mus m 1 and other mammalian proteins. Mus m 1 is most closely related to Rat n 1 (66% identity) and shows limited sequence identity with other members of the family of mammalian lipocalin allergens (eg, Equ c 1, 48%; Bos d 2, 25%; Can f 1, 21%; and Can f 2, 29%). The sequence similarity searches also revealed that Mus m 1 shows 22% identity with lipocalin pheromone carriers (aphrodisins) produced by female black-bellied hamsters and golden hamsters (sequence accession nos. X65238 and AJ225170, respectively).<sup>25</sup> However, we were unable to find any gerbil amino acid sequences that were related to Mus m 1.

### DISCUSSION

Previous studies have shown significant differences between environmental mouse and rat allergen

**TABLE I.** Mus m 1 levels in allergenic products of mammalian origin\*

Allergen manufacturer	Species	Potency (wt/vol)	Mus m 1 (µg/mL)
Greer Laboratories	Mouse epithelia	1:20	2.05
	Gerbil epithelia no. 1	1:20	0.11
	Gerbil epithelia no. 2	1:20	0.04
	Cattle epithelia	1:20	<0.04
	Dog epithelia	1:20	<0.04
	Goat epithelia	1:20	<0.04
	Hog epithelia	1:20	<0.04
	Rabbit epithelia	1:20	<0.04
	Rat epithelia	1:20	<0.04
ALK-ABELLO	Mouse epithelia no. 1	1:20	0.27
	Mouse epithelia no. 2	1:20	0.36
	Cat epithelia	10,000 BAU	<0.04
	Horse epithelia	1:20	<0.04
Allergy Laboratories of Oklahoma	Mouse hair and epithelia	1:50	0.17
	Hamster hair and epithelia	1:20	<0.04
Nelco Laboratories	Mouse epithelia	1:20	10.02
Antigen Laboratories	Guinea pig hair	1:20	<0.04
INDOOR Biotechnologies	Mouse urine	1:20	4550

\*Selected data from each species tested.

measurements of dust or air samples made by means of RAST inhibition, ELISA inhibition, or polyclonal sandwich assays.<sup>17-22</sup> Those studies emphasized the need for improved standardization of assay reagents and allergen standards for optimal exposure assessment. We have developed a polyclonal antibody-based ELISA for accurate analysis and quantification of the major urinary allergen Mus m 1. The assay used antibodies raised against a highly homogeneous preparation of rMus m 1, which had been used to determine the allergen structure by means of nuclear magnetic resonance spectroscopy and x-ray crystallography.<sup>13,15</sup> In the earlier studies polyclonal antibodies raised against rodent urinary allergens showed multiple bands on immunoblotting, which were attributed to aggregated forms of mouse allergen.<sup>22</sup> Our data established that the polyclonal antibodies raised against rMus m 1 reacted with a single Mus m 1 band on immunoblots with mouse urine and confirmed the specificity of the antibodies for binding to the natural mouse allergen. Both ELISA and immunoblotting results showed that the reagents could detect picogram amounts of Mus m 1. The ELISA results also showed a strong correlation between allergen levels in house dust extracts that were measured by using 2 different Mus m 1 standards: purified natural Mus m 1 and an in house preparation of mouse urine that was substandardized against the purified allergen. Comparison of Mus m 1 levels determined by means of ELISA and OD at 276 nm suggested a quantitative difference of approximately 2-fold between the absolute levels of Mus m 1 in both allergen standards, as measured by using the 2 techniques. Similar differences between absolute allergen measurements have been observed for other allergens. For example, the European Union CREATE project comparing protein values for purified natural and recombinant mite, grass pollen, birch, and olive pollen allergens found

differences of 1.5- to 4-fold between protein estimation by use of amino acid analysis, the extinction coefficient, and colorimetric assays.<sup>26</sup> The Mus m 1 data fit well within these values. rMus m 1 appears to be an excellent candidate for standardization purposes and would enable the results of exposure assessments to be directly compared.<sup>15</sup>

The specificity data showed that 10 of 11 allergenic products from other mammalian species showed no reactivity in the Mus m 1 ELISA and that the assay was highly specific. This is the most comprehensive analysis of specificity for rodent allergen assays that has been published to date. The fact that rat allergenic products were negative in the assay is strong evidence of specificity because rat urinary allergen, Rat n 1, shows the highest degree of amino acid sequence identity to Mus m 1 (65%). The only animal extract to show any reactivity, apart from mouse extract, was from the gerbil. The 2 gerbil products that were tested were different lots from Greer Laboratories, and both reacted in the ELISA. Sequence homology searches were used to identify gerbil sequences that were related to Mus m 1. These searches revealed a low level of homology between Mus m 1 and hamster aphrodisins (22%), but these are unlikely to be of allergenic significance because aphrodisins function as pheromone carriers in female mouse vaginal tissue and Bartholin's glands.<sup>25</sup> Aphrodisins show similar homology to Mus m 1 as other lipocalin proteins, including some of the lipocalin allergens (eg, Can f 1 and Can f 2). In phylogenetics the mouse subfamily Murinae (which includes some rat species) is more closely related to gerbils (Gerbillinae) than to hamsters (Cricetinae).<sup>27,28</sup> Gerbils might occasionally cause allergic reactions.<sup>29,30</sup> However, as yet, no gerbil allergens have been defined. Our results raise the possibility the gerbils produce urinary allergens that cross-react with those of mouse and rat. Contamination of gerbil extracts with mouse urine could

explain the results. However, this seems unlikely because we found no evidence for contamination in extracts from other species (Table I).

The data strongly suggest that the Mus m 1 ELISA is suitable for environmental assessment of mouse allergen exposure at home and in the workplace. The assay was validated by using samples that were collected in Baltimore, Md, as part of studies of mouse allergen exposure in children with asthma. Those studies confirmed earlier investigations that showed a high prevalence of Mus m 1 in inner-city homes (74% to 100%), with levels of up to 600 µg/g.<sup>7,8</sup> Our data show that the current assay provides results that are directly comparable in terms of both sensitivity and specificity. The Mus m 1 ELISA also has application to occupational exposure assessment. Recently, we have used the assay to measure airborne occupational exposure to mouse allergen at various locations in the vivarium at the University of Virginia. Air samples were collected in animal rooms, cage-washing areas, and offices with an ion charging device, and the results showed significant differences in exposure levels at the different sites.<sup>31</sup> These results suggest that the Mus m 1 assay will be suitable for environmental monitoring, for assessment of risk levels of exposure, and for evaluation of allergen avoidance devices and procedures.

The CIM of the University of Parma is acknowledged for the use of the mass spectrometer. We are also grateful to Dr Anna Pomés for performing the sequence analyses.

**Note added in proof.** Since this article was accepted for publication, an independent study has reported that the Mus m 1 assay sensitivity could be increased by amplifying ELISA.<sup>32</sup>

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